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Selective serotonin reuptake inhibitors—A new modality for the treatment of lymphoma/leukaemia?

Christian Schuster [a], *Nora Fernbach* [b], *Uwe Rix* [b], *Giulio Superti-Furga* [b], *Marion Holy* [a], *Michael Freissmuth* [a,*], *Harald H. Sitte* [a], *Veronika Sexl* [a]

[a] *Institute of Pharmacology, Center of Biomolecular Medicine and Pharmacology, Medical University of Vienna, Waehringer Str. 13a, A-1090 Vienna, Austria*

[b] *Research Center for Molecular Medicine (CeMM), Austrian Academy of Sciences, Vienna, Austria*

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ABSTRACT

Selective serotonin reuptake inhibitors (SSRIs) have recently been reported to specifically kill malignant cells of B-lymphoid origin, i.e., cells derived from Burkitt lymphoma. Accordingly, SSRIs have been proposed as lead compounds in the development of new approaches to the treatment of lymphoma/leukaemia. Here we attempted to dissect the underlying signaling pathways by comparing susceptible and resistant cell lines. However, we found that all cell lines investigated underwent apoptotic cell death when exposed to SSRI concentrations exceeding 10 μM regardless of whether the cell lines were derived from B- (e.g., Namalwa, Ramos, Daudi, RL7), T-lymphoid tumors (e.g., Molt-4, Jurkat, CCRF-CEM) or other sources. The structure–activity relationship readily distinguished the pro-apoptotic and growth inhibitory effect of SSRIs from their eponymous action (blockage of the serotonin transporter): acetylation of the SSRIs fluvoxamine and paroxetine abrogated the ability of these compounds to inhibit 5HT-uptake, but did not impair their cytotoxic action. Based on these data we conclude that (i) SSRIs inhibit growth of transformed cells, but that (ii) this effect is neither specific for malignant cells nor specific for any particular cellular subset. (iii) The pro-apoptotic effect of SSRIs (at μM concentrations) is unrelated to their principal pharmacological action, i.e., inhibition of serotonin uptake (at nM concentrations). SSRIs or improved versions thereof are therefore unlikely to represent useful lead compounds for inducing apoptosis in B-cell derived tumors: the underlying mechanism is not confined to any specific cell lineage.

1. Introduction

Burkitt lymphoma is endemic in the equatorial malaria regions and is of particular relevance in sub-Saharan Africa, where it is the most common childhood cancer: tumor growth is apparently promoted due to a combination of infections and possibly herbal remedies, i.e., infection with the Epstein-Barr virus, sustained parasite load due to repeated infections with *Plasmodium falciparum*, with one or several arboviruses and the use of extracts from *Euphorbia tirucalli* [1,2]. More recently,

* *Corresponding author*. Tel.: +43 1 4277 64171; fax: +43 1 4277 9641.
E-mail address: michael.freissmuth@meduniwien.ac.at (M. Freissmuth).

Abbreviations: Annexin V-APC-Cy7, annexin V-allophycocyanin-cyanin7; DAT, dopamine transporter; ESI-MS, electron spray ionization mass spectrometry; FACS, fluorescence activated cell sorting; FCS, fetal calf serum; HPLC, high performance liquid chromatography; SERT, serotonin transporter; SSRI, selective serotonin reuptake inhibitors

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there has been a sharp rise in the incidence of Burkitt lymphoma in the industrialized countries due to the HIV epidemic [3].

Burkitt lymphoma is a sad example for a cancer, in which access to treatment can be the limiting factor: when treated with cytotoxic drugs, children with Burkitt lymphoma have a good prognosis. However, in many instances in sub-Saharan Africa, the cytotoxic drug regimen and the associated supportive medical care are beyond the financial means of many families. Accordingly, parents withdraw their children from therapy or withhold treatment [4]. In this situation, an alternative, less expensive treatment would be a major breakthrough. Hopes have been raised by the report that lymphoid cells express the serotonin transporter, which may be targeted to elicit apoptosis in Burkitt lymphoma [5]. In fact, selective serotonin reuptake inhibitors (SSRIs) were reported to specifically induce apoptosis in Burkitt lymphoma cells [6]: this effect was described as absent in non-transformed quiescent B-lymphoid cells or cells of other origin. In their most recent work, Meredith et al. [7] extended these earlier observations to additional drugs targeting serotonin uptake and to additional B-lymphoid cell types including multiple myeloma cell lines. These investigations also suggested that SSRIs elicited this pro-apoptotic effect by a mechanism unrelated to their ability to block the serotonin transporter.

In the present work, we followed up on this innovative concept and designed experiments intended to characterize the relevant signaling pathway(s). We searched for susceptible and resistant cell lines, the underlying rationale being that susceptible and resistant cells ought to differ by the presence or absence of one or several crucial components. However, while SSRIs reproducibly induced an apoptotic response in B-lymphoid tumor cells, we did not find this effect to be confined to B-lymphoid cells or tumor cells. In fact, we failed to identify a single cell line that tolerated SSRI treatment within the concentration range relevant for SSRI-induced apoptosis in malignant cells.

2. Materials and methods

2.1. Materials

FCS and media (Dulbecco's modified Eagle medium (DMEM), RPMI-1640, non-essential amino acids) were from PAA Laboratories (Linz, Austria). Radioactive compounds ([^{3}H]serotonin, [^{3}H]thymidine) were from New England Nuclear (Dreieich, Germany). The BD-Pharmingen Annexin V-PE Apoptosis Detection Kit I was purchased from BD Biosciences (Heidelberg, Germany), scintillation fluid (Rotiszint ECO plus) was from Roth (Karlsruhe, Germany). Selective serotonin reuptake inhibitors and β-mercaptoethanol were obtained through Sigma–Aldrich (St. Louis, MO). All other chemicals were analytical grade.

2.2. Cell lines, primary cells and tissue culture

The following commercially available cell lines were obtained from ATCC (Manassas, VA; ATCC number in brackets): RL-7 (ATCC no. CRL-2261), REH (ATCC no. CRL-8266), Daudi (ATCC no. CCL-213), Namalwa (ATCC no. CRL-1432) and Ramos (ATCC no. CRL-1596) cell lines are established human B-lymphoid cell lines; Molt-4 (ATCC no. CRL-1582), CCRF-CEM (ATCC no. CCL-119), Jurkat (ATCC no. TIB-152), Mac-2A [8], HPB-ALL [9] and SU-DHL-1 [10] cells are transformed human T cell lines. EL-4 (ATCC no. TIB-39) is a murine T cell lymphoma line and K562 (ATCC no. CCL-243) is a well-defined human erythroleukaemia cell line transformed by the bcr/abl oncogene.

The v-abl cell line is a murine cell line, which was established in our laboratory by infecting bone marrow cells of a B6/C57 mouse with a retrovirus encoding the v-abl oncogene [11]. The Eμ-myc cell line is a murine cell line which was established from the bone marrow of a diseased Eμ-myc transgenic mouse [12]: the mouse was sacrificed; the femurs were flushed with sterile RPMI 1640 medium and the resulting bone marrow cell suspension was placed in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin and 5 μM β-mercaptoethanol at 37 °C under an atmosphere of 5% CO_2. After several weeks, stably growing cell lines emerged, which were subcloned and used in the experiments. To obtain fresh Eμ-myc tumor cells, mice carrying the Eμ-myc transgene were sacrificed, when they were visibly affected by the disease; tumors (inguinal lymph nodes) were excised, minced and pressed through a 70 μm cell strainer to create a cell suspension, which was propagated in cell culture.

Preparation of mouse embryo fibroblasts (MEF): embryos (E12.5) were harvested from pregnant C57B6/J females who had been allowed to mate with C57B6/J males. Fetal livers were removed and the remaining embryos were cut into small pieces, which were trypsinized for 1 h at 37 °C. The resulting cell suspension was placed into DMEM culture medium supplemented with 10% FCS, 2 mM glutamine, non-essential amino acids, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C under an atmosphere of 5% CO_2. At confluence, the cells were detached and passaged. Cells of 3rd to 4th passage were used in the experiments designed to assess the cytotoxic action of SSRIs and related compounds (see below).

Non-adherent cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin and 5 μM β-mercaptoethanol at 37 °C under an atmosphere of 5% CO_2. HEK293 cells expressing the human transporters for serotonin [13] and dopamine [14], CHO cells and NIH3T3 cells were maintained in DMEM medium supplemented with 10% FCS (PAA), 2 mM glutamine, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin. HEK293 cells expressing the human SERT were transfected with a plasmid driving the expression of human neuronal NOS-synthase (nNOS) [15] using Lipofectamine Plus (Invitrogen, Karlsbad, CA) resulting in transfection efficiencies >90%.

2.3. [^{3}H]Thymidine incorporation assay

Cells (10^5 and 10^4 cells/well for non-adherent and adherent cells, respectively) were seeded in 96 well plates and incubated with the compounds as indicated in the figure legends. [^{3}H]Thymidine was added to a final concentration of 0.1 μCi/well. The acetylated SSRIs had to be dissolved in DMSO. For these experiments, controls containing DMSO alone were performed in addition to exclude any non-specific cytotoxic

effects unrelated to the effects of SSRI treatment. After a 24 h incubation period the cells were rapidly frozen, thawed and subsequently analyzed using a Skatron semiautomatic cell harvester. In some instances, cells were incubated for up to 72 h in the presence of 6 logarithmically spaced concentrations (2 concentrations/order of magnitude) of paroxetine and of citalopram; [^{3}H]thymidine was added during the last 24 h and the reaction was terminated by freezing as outlined above. The amount of incorporated [^{3}H]thymidine was determined in a Packard scintillation counter after addition of scintillation fluid.

2.4. FACS analysis

Cells (5×10^4 and 5×10^5 cells/well for adherent and non-adherent cells, respectively) were seeded in a 24 well plate and the compounds were added at the concentrations indicated in the figure legends. After 24 h, the cells were harvested and stained with antibodies against annexin V according to the instructions of the manufacturer. Cells, in which the barrier function of the membrane had broken down (necrotic cells/late apoptotic cells), were detected by staining with propidium iodide (final concentration = 1 mg/l). Cells were analyzed using a BD FACS Canto II and BD FACS Diva software.

2.5. Serotonin-uptake experiments

HEK293 cells (10^5 cells/well) expressing the human transporters for serotonin (13) and dopamine (14) were seeded in 48 well plates. Twenty four hour thereafter the cells were washed with Krebs Hepes Buffer (KHB) containing 10 mM HEPES, 130 mM NaCl, 1.3 mM KH_2PO_4, 1.5 mM $CaCl_2{\cdot}2H_2O$, 0.5 mM $MgSO_4{\cdot}7H_2O$, 34 mM glucose, final pH 7.35. Subsequently, the cells were pre-incubated for 5 min in 0.1 ml KHB containing the indicated concentrations of SSRIs or their acetylated derivatives. Then, the fluid was replaced by KHB containing the indicated concentrations of SSRIs and, in addition, 0.05 μM [^{3}H]serotonin. After 1 min, the cells were washed with ice cold KHB and lysed by adding 500 μl of 1% SDS containing solution. The lysed cells were transferred into 5 ml scintillation fluid and [^{3}H]serotonin uptake was determined using a Packard scintillation counter.

2.6. Synthesis, analysis and purification of acetylated SSRIs

The respective SSRIs (~5 μmol) were dissolved in 0.5 ml acetonitrile (abs.) and 1.5 equivalents (~7.5 μmol) of acetic acid anhydride as well as 1.5 equivalents (~7.5 μmol) of triethylamine were added. The reaction mixture was stirred for 16 h at room temperature under argon atmosphere. The acetylated SSRIs were subsequently analyzed by HPLC–MS (on a Alliance HT-HPLC equipped with a diode array detector and a ZQ mass spectrometer equipped with an electrospray probe, Waters; Manchester, UK). The yield of the reaction was close to 100% (*N*-acetyl-paroxetine: 98%, *N*-acetyl-fluvoxamine: 99%). Compounds were extracted with ethyl acetate and further characterized by ^{1}H NMR spectroscopy (DRX400 NMR spectrometer, Bruker). ^{1}H NMR (400 MHz, $CDCl_3$) for *N*-acetyl-fluvoxamine: δ = 7.67 (d, J = 10 Hz, 2H), 7.55 (d, J = 10 Hz, 2H), 6.04 (s [br], 1H, C═ONH), 4.21 (t, $J_1 = J_2$ = 5 Hz, 2H, 1″-H_2), 3.53 (dt, $J_1 = J_2$ = 5 Hz, 2H, 2″-H_2), 3.34 (t, J = 3 Hz, 2H, 5-H_2), 3.25 (s, 3H, OCH_3), 2.73 (t, J = 6 Hz, 2H, 2-H_2), 1.91 (s, 3H, C═OCH_3), 1.51–1.60 (m, 4H). ^{1}H NMR (400 MHz, $CDCl_3$) for *N*-acetyl-paroxetine: δ = 7.04 (dd, J_1 = 9 Hz, J_2 = 6 Hz, 2H, 2″-H_2), 6.93 (dd, $J_1 = J_2$ = 9 Hz, 2H, 3″-H_2), 6.57 (d, J = 10 Hz, 1H, 3′-H), 6.28 (d, J = 1 Hz, 1H, 6′-H), 6.08 (dd, J_1 = 10 Hz, J_2 = 1 Hz, 1H, 4′-H), 5.81 (s, 2H, O–CH_2–O), 4.64–4.94 (m, 1H), 3.78–4.18 (m, 1H), 3.54 (dd, J_1 = 9 Hz, J_2 = 2 Hz, 1H, 7-H_β), 3.42 (dd, J_1 = 9 Hz, J_2 = 8 Hz, 1H, 7-H_α), 2.97–3.16 (m, 1H), 2.54–2.78 (m, 2H), 2.09 (s, 3H, C═OCH_3), 1.75–2.02 (m, 2H).

2.7. Immunoblotting for Bcl-2

Cells were lysed in ice cold lysis buffer [composition in mM: 10 Tris/HCl (pH 7.5), 50 NaCl, 30 $NaPP_i$, 50 NaF, 2 EDTA, 1 PMSF, 0.1 sodium orthovanadate; 1% (w/w) Triton X100, 3 μg/ml each aprotinin, 0.5 μg/ml leupeptin and 1 μg/ml pepstatin]. Undissolved material was removed by centrifugation (15 min at 20,000 × g) at 4 °C and protein content in the supernatant was quantified by the bicinchonic acid method using the BCA kit as recommended by the manufacturer (Pierce, Rockford). Proteins (50 μg/lane) were electrophoretically separated on polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE), subsequently transferred to nitrocellulose membrane (Millipore, Bedford, MA) and probed with antibodies against Bcl-2 (SC-7382, Santa Cruz Biotechnology) and β-actin as loading control (A2228, Sigma; St. Louis, MO). Immunoreactive bands were visualized by chemoluminiscent detection (ECL detection kit, Amersham) using protein A-conjugated horseradish peroxidase (Amersham, Arlington Heights, UK). Exposed films were scanned and band density was quantified using ScionImage software.

2.8. Data analysis

Data were subjected to curvilinear regression using the equation for a three-parameter logistic function (=Hill equation) to calculate IC_{50} values using the software implemented in Graph Pad Prism. Data presented are means ± S.E.M. of three independent experiments performed in triplicate.

3. Results

3.1. Paroxetine inhibits [^{3}H]thymidine incorporation in B- and T-lymphoid cell lines

We employed [^{3}H]thymidine incorporation as an assay to screen a panel of cell lines for their susceptibility to the cytotoxic action of the SSRIs. This assay offers several advantages that make it suitable for a screening procedure, namely large sample throughput, high sensitivity, reproducibility and a quantitative readout which facilitates the determination of IC_{50} values. The cells were incubated for 24 h with paroxetine (and other SSRIs) in the concentration range of 1–300 μM and [^{3}H]thymidine incorporation was determined. Our initial aim was to identify a minimum of two cell lines of B-lymphoid origin, one which was highly susceptible to the action of SSRIs and another one resistant to their cytotoxic effect. We surmised that the availability of

these two cell lines was likely to increase the probability of identifying the components relevant to the action of SSRIs. Seven malignant B-cell lines (five human and two murine cell lines) were tested: paroxetine inhibited S-phase entry in all of these cells with IC_{50} values ranging from 5 to 20 μM (Fig. 1A, Table 1). Based on these observations, we concluded that all cell lines of B-lymphoid origin were susceptible to paroxetine. Hence, we extended our search for a suitable non-responding control cell line and screened cell lines of T-lymphoid origin, because we reasoned that these were the closest relatives to B-lymphoid cells. Again, paroxetine completely blocked [^{3}H]thymidine incorporation in seven independent human T-lymphoid cell lines with IC_{50} values ranging from 15 to 120 μM (Fig. 1B, Table 1). It is evident from Table 1 that – on average – cells of T-lymphoid origin were somewhat less susceptible than cells of B-lymphoid origin. However, these differences were modest; we therefore concluded that the cytotoxic action of SSRIs was not restricted to cells of B-cell origin, but it was clearly also seen with transformed cells of T-lymphoid origin. We also tested additional SSRIs for their cytotoxic action. Table 1 summarizes the pertinent data set for citalopram: while citalopram was less potent than paroxetine in inhibiting [^{3}H]thymidine incorporation, all cell lines – tested irrespective of their lineage determination – were susceptible to citalopram treatment within a comparable concentration range. Cytotoxicity typically shows hysteresis, because a threshold has to be overcome: high concentrations may be tolerated for short incubation periods and upon prolonged incubation, less susceptible cells will eventually also become more susceptible. In other words, the IC_{50} for growth inhition/EC_{50} for apoptosis induction may also depend on the incubation time. We tested this assumption by comparing IC_{50} values determined after a 24 h incubation with paroxetine with those observed after a 48 and 72 h incubation: regardless of whether we used a cell line of B (Daudi) or T-lymphoid (CCRF) or myeloid origin (K562), prolonged incubations increased the susceptibility of cells to the inhibitory action of paroxetine (Fig. 1C).

3.2. *SSRIs induce apoptosis in B-, T-lymphoid and in myeloid cells*

[^{3}H]Thymidine incorporation quantifies cell proliferation; cell death is only indirectly detected as a decline in [^{3}H]thymidine incorporation. We confirmed that SSRIs induced apoptosis in the concentration range in which they inhibited [^{3}H]thymidine incorporation by monitoring one of the earliest events triggered during programmed cell death, namely the breakdown of the

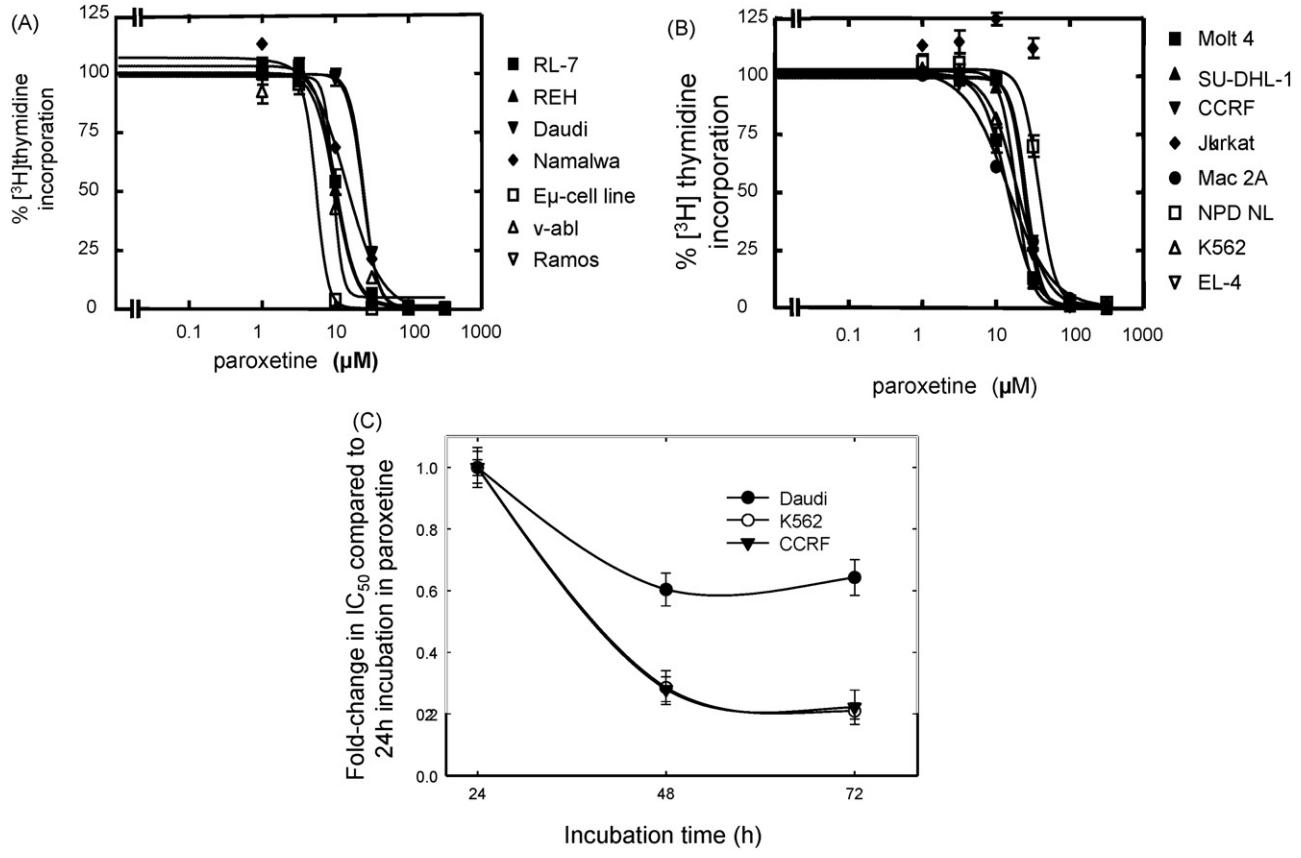

Fig. 1 – Inhibition by paroxetine of [^{3}H]thymidine incorporation by transformed lymphoid cell lines. The indicated transformed B-cell lines (A) and T-cell lines (B) were seeded on 96 well plates (10^5 cells/well) and incubated with increasing concentrations of paroxetine and 0.1 μCi [^{3}H]thymidine/well for 24 h. The solid lines were drawn by fitting the data to a three-parameter logistic equation. To analyze the time-dependent change in susceptibility (C) cells were incubated for 24, 48 and 72 h in six logarithmically spaced concentrations of paroxetine or citalopram; 0.1 μCi [^{3}H]thymidine/well was added for the last 24 h. Data represent means ± S.E.M. (n = 3).

Table 1 – IC_{50} values of paroxetine and citalopram for inhibition [^{3}H]thymidine incorporation by transformed B- and T-lymphoid cell lines and the myeloid cell line K562

Cell line	Paroxetine IC_{50} (μM)	Citalopram IC_{50} (μM)
B-lymphoid		
RL-7	12.27 ± 1.28	88.3 ± 13.0
REH	10.0 ± 1.25	136.6 ± 14.3
Daudi	23.8 ± 1.35	160.1 ± 13.6
Ramos	24.5 ± 1.37	79.1 ± 12.8
Eμ-myc murine cell line	5.5 ± 1.46	77.9 ± 14.9
v-abl murine cell line	9.5 ± 1.30	90.0 ± 12.2
Namalwa	14.3 ± 1.21	94.8 ± 11.9
T-lymphoid		
SU-DHL-1	18.7 ± 1.41	207.1 ± 14.7
MOLT-4	14.6 ± 1.26	117.2 ± 12.9
CCRF	25.7 ± 1.33	149.4 ± 13.8
EL-4	24.1 ± 1.36	118.6 ± 12.9
Jurkat	116.3 ± 17.9	185.7 ± 13.1
Mac-2A	14.7 ± 1.15	72.9 ± 13.6
HPD-HL	38.07 ± 1.34	185.1 ± 14.9
K562 (myeloid)	19.8 ± 1.22	202.3 ± 12.6

Cells were treated as outlined under Section 2 and in the legend to Fig. 1. IC_{50} values were calculated by non-linear least squares curve fitting of the data to the Hill-equation. Data are means ± S.E.M. (n = 3).

asymmetric phospholipid distribution and the accumulation of phosphatidylethanolamine and phosphatidylserine on the outer leaflet of the plasma membrane. Accumulation of phosphatidylserine can be readily visualized by staining with annexin V (which binds specifically to phosphatidylserine). We incubated B and T cell lines as well as myeloid cells in the presence of paroxetine and quantified the amount of cell surface-associated annexin V by FACS. All cell lines tested were unequivocally positive for annexin V when treated with paroxetine concentrations corresponding to the IC_{50} range determined by [^{3}H]thymidine incorporation. Representative dot plots are shown in Fig. 2 and concentration–response curves are shown in Fig. 3C. It is also evident from the dot plots shown in Fig. 2 that in all instances, staining for annexin V was seen at lower concentrations than uptake of propidium iodide, which labels late apoptotic as well as necrotic cells. The induction of apoptosis was also readily detectable by light microscopy. The cells treated with paroxetine displayed typical morphological changes indicative of apoptosis including cell shrinkage, membrane blebbing, increased granularity and nuclear fragmentation (not shown).

3.3. *The pro-apoptotic effect of SSRIs is also seen in non-transformed cells*

We next reasoned that responsiveness to SSRIs might be a feature of transformed cells and that non-transformed cells or cells of non-hematological origin were resistant to the pro-apoptotic effects of SSRIs. To address this issue we tested the following cells: (i) CHO cells—transformed hamster ovary cells with a fibroblast phenotype [16]; (ii) HEK293 cells—adenovirally transformed human fibroblast-like cells [17]; (iii) mouse embryonic fibroblasts (MEFs)—a primary non-transformed fresh cell preparation; (iv) NIH3T3 fibroblasts—non-transformed immortal cells; (v) fresh murine splenocytes—primary non-transformed cells; (vi) fresh tumor cells derived from Eμ-myc mice. Again, we used paroxetine and citalopram and analyzed the response of this cell population in [^{3}H]thymidine incorporation and annexin V assays. The results are summarized in Fig. 3A and Table 2. Briefly, paroxetine and citalopram inhibited DNA-synthesis in all cell lines regardless of their phenotype (transformed, non-transformed, established cell line or primary cell culture) with IC_{50}-values that were within the range seen with cell lines of lymphoid origin (*cf.* Table 1). We confirmed by staining for phosphatidylserine with annexin V that SSRIs induced apoptosis in these cells: a representative experiment with HEK293 cells is shown in Fig. 3B and the resulting concentration–response curve is plotted in Fig. 3C.

3.4. *Bcl-2 levels do not explain the variation in susceptibility to SSRI*

The variable susceptibility of Burkitt lymphoma cell lines to SSRIs was reported to be accounted for by differences in the expression of the antiapoptotic protein Bcl-2 [6]. It is evident from Table 1 (*cf.* murine Eμ-myc transformed B-cells versus transformed human Jurkat T cells) that there was an up to 20-fold variation in the concentration of SSRIs required to inhibit [^{3}H]thymidine incorporation. Accordingly, we determined Bcl-2 levels in nine selected cell lines by immunoblotting and densitometry and tested, if the low levels of Bcl-2 were predictive of enhanced responsiveness to SSRIs: Bcl-2 levels (plotted on the *x*-axis of Fig. 4) varied up to 10-fold. However, it is evident that there was not any appreciable correlation between the levels of Bcl-2 and the IC_{50} of SSRIs.

3.5. *The cytotoxic effect of SSRIs in HEK cells does not depend on SERT expression*

Bone marrow cells are known to express monoamine transporters and this is also true for lymphocytes [18]. HEK293 cells and MEFs do not express SERT; nevertheless these cells underwent apoptosis when challenged with SSRIs. Based on

Table 2 – IC_{50} values of paroxetine and citalopram for inhibition [^{3}H]thymidine incorporation by fibroblast cell lines, primary cells and untransformed cells

Cell line	Paroxetine IC_{50} (μM)	Citalopram IC_{50} (μM)
HEK 293	25.5 ± 1.30	157.3 ± 14.8
CHO	13.5 ± 1.29	49.6 ± 14.1
NIH3T3	30.2 ± 1.39	122.7 ± 13.6
MEF	46.1 ± 5.3	35.1 ± 13.3
Primary splenocytes	14.2 ± 1.38	93.6 ± 15.7
Primary Eμ-myc tumor cells	8.8 ± 1.30	29.6 ± 12.2

Cells were treated as outlined under Section 2 and in the legend to Fig. 3. IC_{50} values were calculated by non-linear least squares curve fitting of the data to the Hill-equation. Data are means ± S.E.M. (n = 3).

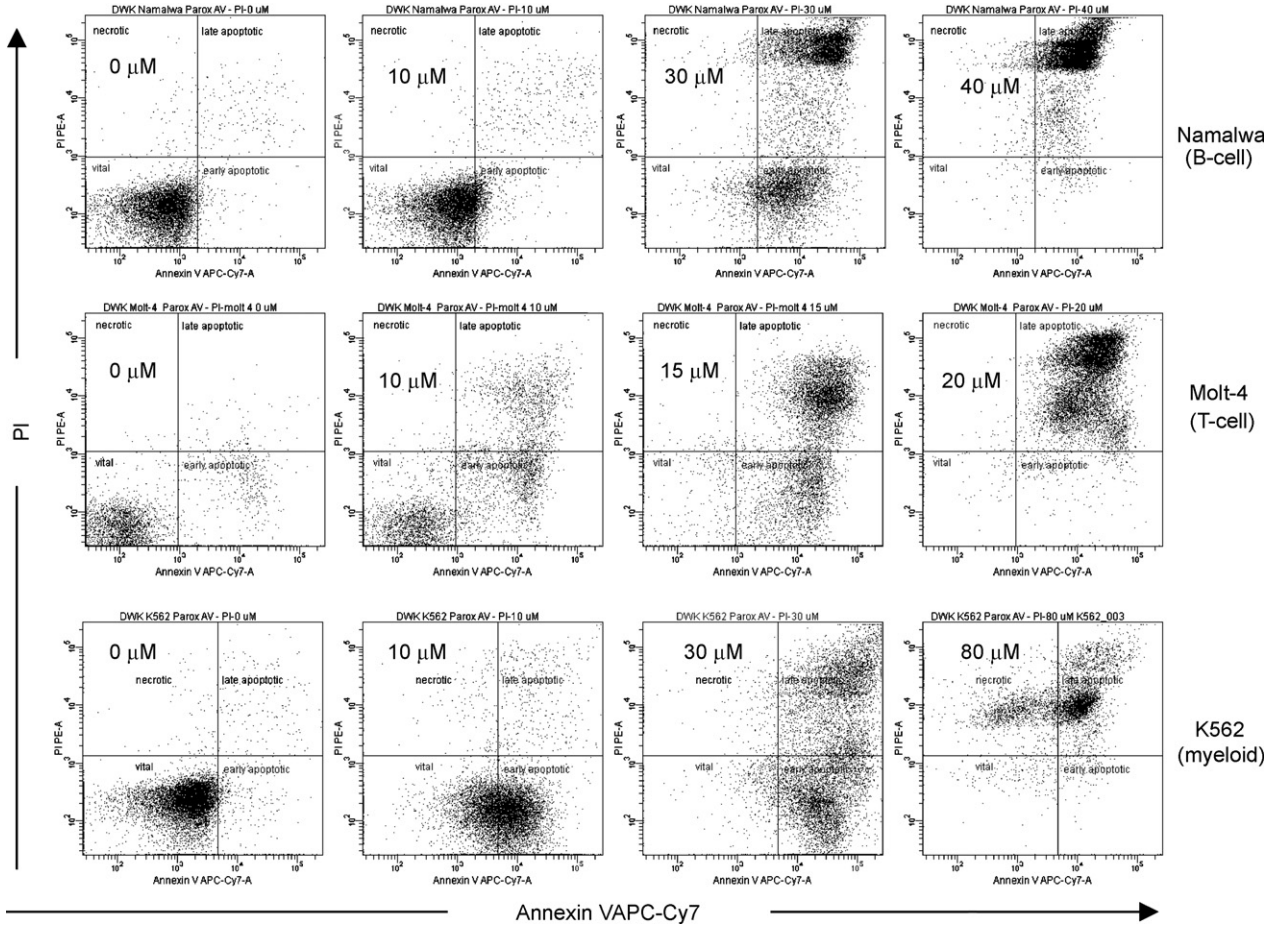

Fig. 2 – Induction of apoptosis by paroxetine in a representative cell line of each, B, T and myeloid origin. B-lymphoid Namalwa (top row). T-lymphoid MOLT-4 (middle row) and myeloid K562 cells were seeded in 24 well plates (5×10^5 cells/well) and incubated with increasing concentrations of paroxetine as indicated. After 24 h, the cells were stained with APC-Cy7-labeled annexin V and incubated in the presence of 1 mg/l propidium iodide (PI). Representative dot plots are shown which correspond to incubations carried out in parallel.

the original observations of Serafeim et al. [5,6], it is conceivable that the presence of SERT enhances the responsiveness of cells to SSRI-induced apoptosis. We therefore used stably transfected HEK293 cell clones expressing SERT under the control of a TET-on promoter [13] and compared them to maternal cells transfected with the empty vector alone. As an additional more suitable control, we also used cell lines stably expressing the dopamine transporter [14]. The presence or absence of SERT did not affect the ability of paroxetine to suppress DNA-synthesis (Fig. 5A; $IC_{50} = 12.5 \pm 1.8$ and 14.3 ± 3.3 μM, for HEK293 cells expressing SERT and DAT, respectively). In addition, we verified that cells underwent SSRI-induced apoptosis over a similar concentration range regardless of whether expression of SERT was repressed in the absence doxycycline or induced by addition thereof (Fig. 5B): it is evident that paroxetine induces the appearance of early (i.e., annexin V-positive in Fig. 5B) and late (i.e., annexin V- and propidium iodide double positive cells in Fig. 5B) over an identical concentration range in uninduced cells (Fig. 5B, upper row) and cells expressing SERT (Fig. 5B, lower row). SERT interacts physically with neuronal NO-synthase [15]. We thus explored a possible contribution of this interaction to SSRI-induced cytotoxicity by co-expressing nNOS and SERT in HEK293 cells. The presence of NOS did neither change the slope of the concentration–response curve nor the IC_{50} of paroxetine (data not shown).

3.6. *Acetylated SSRI loose the ability to inhibit serotonin uptake, but still induce apoptosis*

Given the fact that SERT expression is not a prerequisite for SSRIs to induce cell death (as in HEK cells and MEFs), it was questionable that the pro-apoptotic property of SSRIs was linked to their action on SERT. This interpretation was tested by modifying SSRIs at positions known to be critical for their binding to the serotonin transporter. We selected fluvoxamine and paroxetine because these compounds have primary and secondary amino groups, respectively, which are readily accessible to modification and which are likely to be relevant for supporting the interaction of SSRIs with their target, i.e., SERT [19]. In fact, if the analogous nitrogen is methylated in compounds related to paroxetine, the affinity for SERT drops substantially [20]. We therefore acetylated paroxetine and

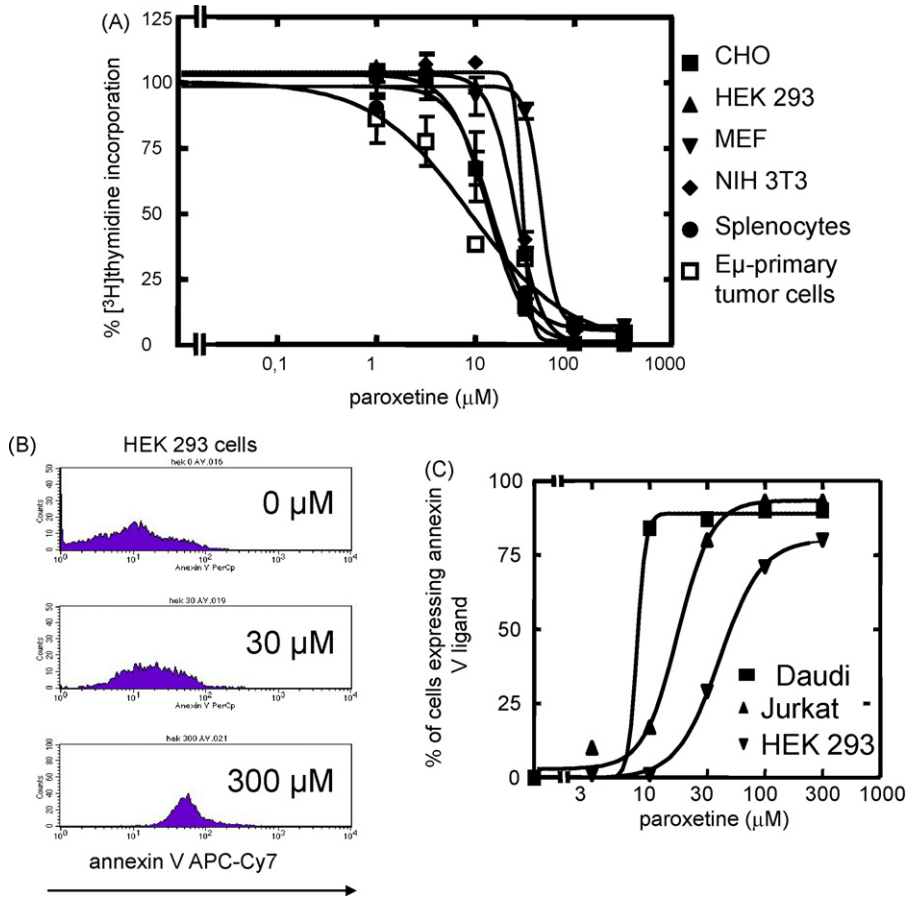

Fig. 3 – Inhibition by paroxetine of [^{3}H]thymidine incorporation by primary cells and by fibroblast cell lines (A) and induction of apoptosis in HEK293 cells (B and C) and a representative B- and T-cell line (C). (A) The indicated cell lines and primary cells were seeded in 96 well plates (10^5 cells/well) and incubated with increasing concentrations of paroxetine and 0.1 μCi [^{3}H]thymidine/well. Data shown represent means ± S.E.M. ($n = 3$). (B) HEK293 cells (5×10^5 cells/well) were incubated with the indicated concentrations of paroxetine. After 24 h, cells were stained with PE-labeled annexin V, which was quantified by FACS. Representative histograms are shown which correspond to incubations carried out in parallel. (C) Representative concentration–response curves for paroxetine-induced increase in annexin V-positive cells. Assay conditions were as outlined for panel B (HEK293 cells) and in the legend to Fig. 2 (for Daudi and Jurkat cells).

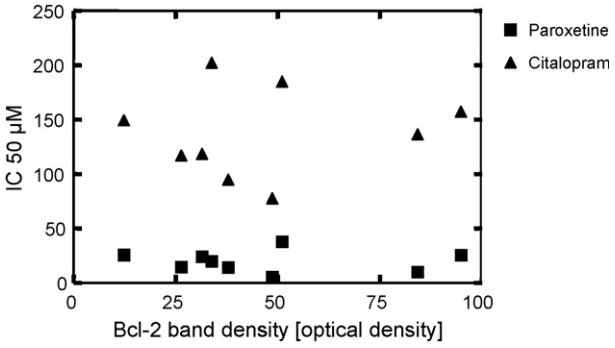

Fig. 4 – Bcl-2 expression levels in different cell lines do not correlate with IC_{50} values. Nine different cell lines were analyzed for their Bcl-2 expression levels by immunoblotting. Optical densities of scanned bands were quantified and related to the IC_{50} values obtained for inhibition of [^{3}H]thymidine incorporation by citalopram and paroxetine. The absence of any significant correlation is evident.

fluvoxamine using acetic acid anhydride (reaction scheme outlined in Fig. 6A for fluvoxamine). The acetylated compounds were characterized by ^{1}H NMR spectroscopy (see Section 2) and HPLC followed by mass spectrometry (shown in Fig. 6B for *N*-acetyl-fluvoxamine).

Acetylation greatly impaired the ability of both, fluvoxamine (Fig. 6C) and paroxetine (Fig. 6D), to inhibit SERT. In contrast, the N-acetylated derivatives did still effectively suppress [^{3}H]thymidine incorporation (shown for the Eμ-myc tumor cell line in Fig. 7): fluvoxamine and acetyl-fluvoxamine acetate were equipotent in inhibiting S-phase entry of cells (Fig. 7A). Interestingly, acetylation of paroxetine somewhat impaired the ability of the resulting *N*-acetyl-paroxetine to suppress [^{3}H]thymidine incorporation; accordingly there was a shift in the IC_{50} values from 6.5 ± 1.4 μM for the parent compound to 93.3 ± 15.2 μM for *N*-acetyl-paroxetine. However, this shift of ~15-fold was modest by comparison to that seen when comparing the affinity of *N*-acetyl-paroxetine and of paroxetine for SERT (>1000-fold difference in IC_{50}, *cf.* Fig. 6C).

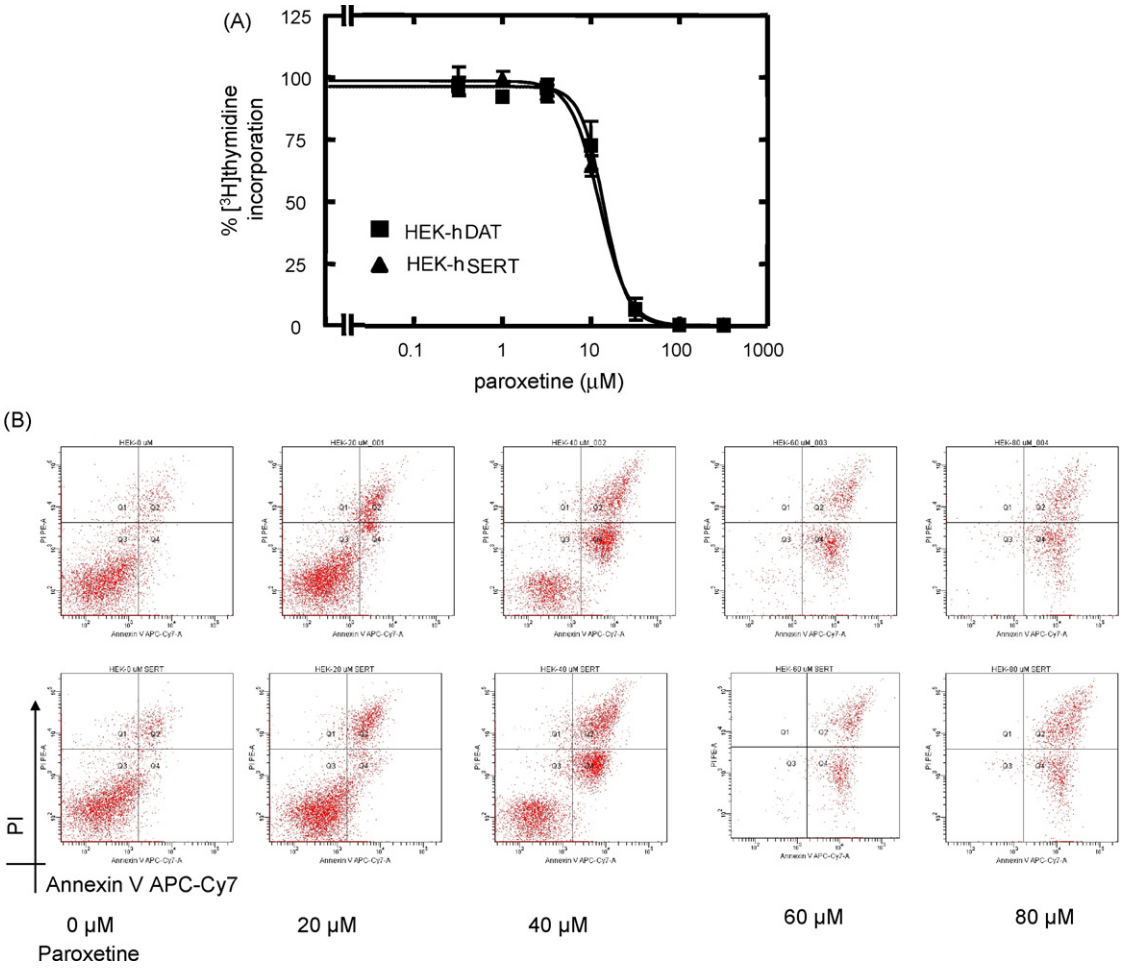

Fig. 5 – Inhibition by paroxetine of [^{3}H]thymidine incorporation (A) and induction of apoptosis (B) by HEK293 cells containing or lacking serotonin transporters. (A) HEK293 cells (10^4/well) expressing either the human serotonin (hSERT, triangles) or dopamine (hDAT, squares) transporter were seeded in 96 well plates and incubated with the indicated concentrations of paroxetine and 0.1 μCi [^{3}H]thymidine/well for 24 h. Data shown represent means ± S.E.M. from (n = 3). (B) HEK293 cells (10^4/well) not expressing the human serotonin (top row) or expressing it (lower row) were seeded on 24 wells and incubated with increasing concentrations of paroxetine as indicated. After 24 h, the cells were stained with APC-Cy7-labeled annexin V and incubated in the presence of 1 mg/l propidium iodide (PI). Representative dot plots are shown which correspond to incubations carried out in parallel.

4. Discussion

Since the original observation that serotonin killed Burkitt lymphoma cells [5], several arguments have been put forth to suggest that targeting SERT was an interesting new modality in the treatment of Burkitt lymphoma and other tumors of B-lymphoid origin: (i) expression of SERT is increased in proliferating B-lymphoid cells and the expression shows a positive correlation with the proliferative capacity [7]. (ii) Serotonin [5] and other SERT-substrates such as methylene-dioxy-metamphetamine (MDMA, ecstasy) and D-fenfluramine inhibit proliferation of B-cell lymphoma cell lines [7]. (iii) SSRIs also suppress proliferation of Burkitt lymphoma cells [6] and other B-lymphoid tumor cells [7]. The similar effect of SERT substrates and SERT inhibitors is difficult to reconcile with a common mechanism of action on SERT. Similarly, the concentrations required for growth inhibition by SSRI is three orders of magnitude higher than that required for inhibition of SERT. This discrepancy strongly argued for the existence of an additional target for SSRIs [7]. (iv) However, the observation that normal lymphocytes are spared from the cytotoxic action of serotonin [5] and SSRIs [6] indicates that either SERT or this hypothetic additional target may represent an Achilles' heel of B-lymphoid tumor cells and hence be selectively exploited to develop a new therapeutic approach.

Our observations, however, refute this concept: (i) we found that many different cell lines and primary cells were susceptible regardless of whether they were of lymphoid origin, virally transformed, immortalized or obtained *ex vivo*. (ii) The cytotoxic effect of SSRIs was unrelated to the presence or absence of SERT and the structure–activity relationship differed from that required for inhibition of SERT. This was most readily evident upon introducing an amide functionality into fluvoxamine and paroxetine. (iii) The concentration–response curves for both, inhibition of [^{3}H]thymidine incorporation and induction of apoptosis, were very steep indicating a threshold phenomenon.

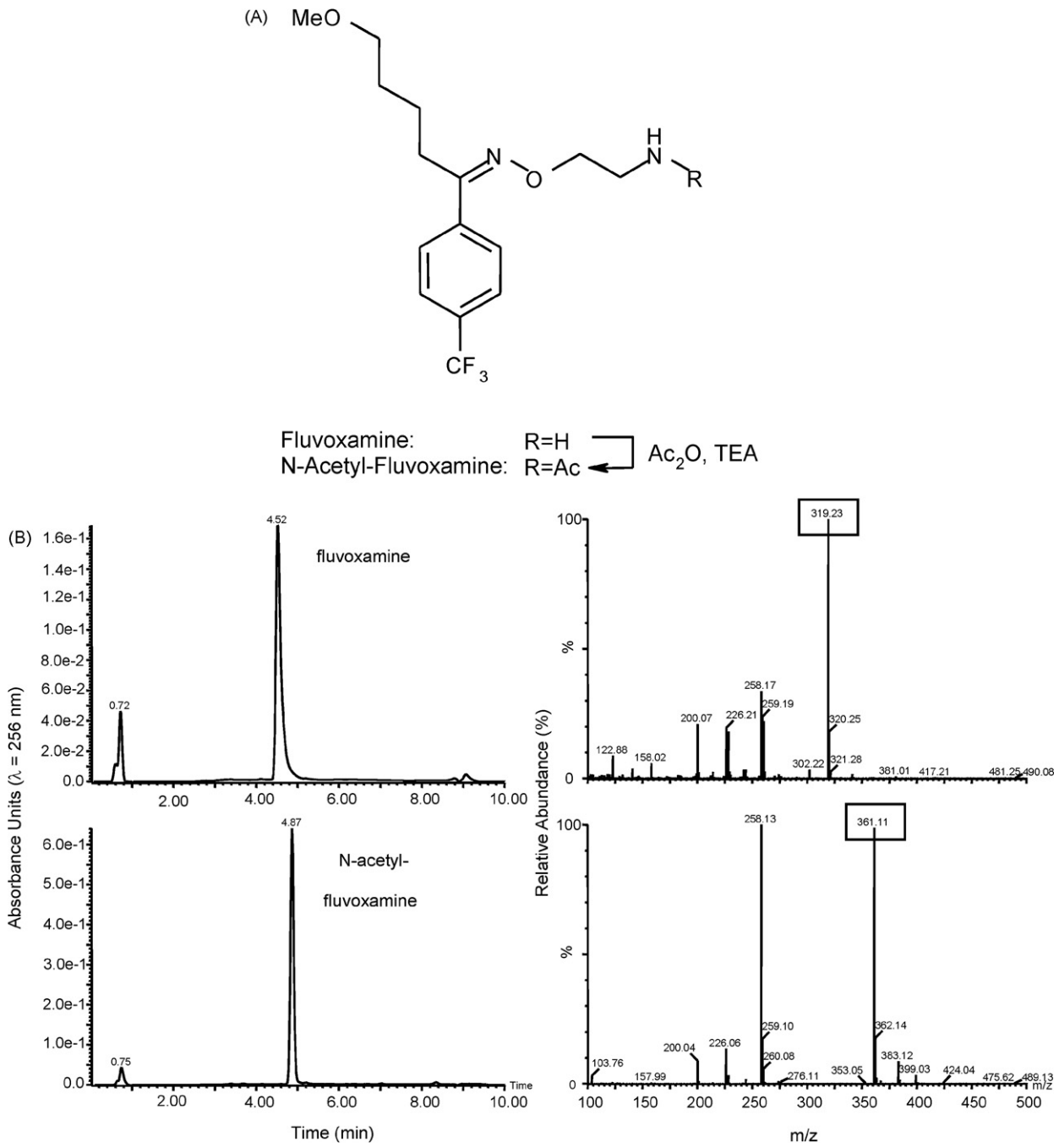

Fig. 6 – Acetylation of fluvoxamine (A and B) abrogates its ability to inhibit serotonin uptake (C). (A) Reaction scheme and structure of fluvoxamine and *N*-acetyl-fluvoxamine (Ac_2O: acetic acid anhydride, TEA: triethylamine). The structure was confirmed by ^1H NMR spectroscopy (see Section 2). (B) Left hand panels: HPLC chromatogram for fluvoxamine (top) and *N*-acetyl-fluvoxamine (bottom); an aliquot corresponding ~2 nmol was applied onto a 3 mm × 50 mm XTerra C18 column (Waters, Milford, MA, USA); the following solvent system was employed: solvent A—aqueous solution of 5 mM NH_4HCO_3 (pH 7.8), solvent B—acetonitrile; flow rate 0.5 ml/min; 10% B at 0 min to 100% B at 6 min (linear gradient). UV chromatograms are displayed at λ = 256 nm. The peak at 0.7 min corresponds to the injection artifact. The identity of the peaks at 4.5 (top) and 4.8 min (bottom) was confirmed by mass spectrometry (right hand panels): mass/charge ratios measured for fluvoxamine (top) and the acetylated reaction product (bottom). Boxed numbers indicate protonated molecule ions ($[M+H]^+$). Fluvoxamine {*m/z* = 319 $[M+H]^+$ (set 100%), 258 $[M–C_2H_6NO^-]^+$ (35% of highest peak)}. *N*-Acetyl-fluvoxamine {*m/z* = 361 $[M+H]^+$ (95%), 399 $[M+K]^+$ (4%), 383 $[M+Na]^+$ (8%), 258 $[M–C_4H_8NO_2^-]^+$ (set 100%)}. Note the complete absence of the species at *m/z* = 319 in the lower panel, which indicates the complete absence of fluvoxamine in the *N*-acetyl-fluvoxamine sample. (C) HEK293 cells (10^5 cells/well) expressing the human SERT were seeded in 24 well dishes and incubated for 5 min with fluvoxamine or *N*-acetyl-fluvoxamine. The uptake reaction was started by addition of $[^3H]$serotonin; after 1 min, the cells were rinsed and lysed. Data shown represent means ± S.E.M. (*n* = 3).

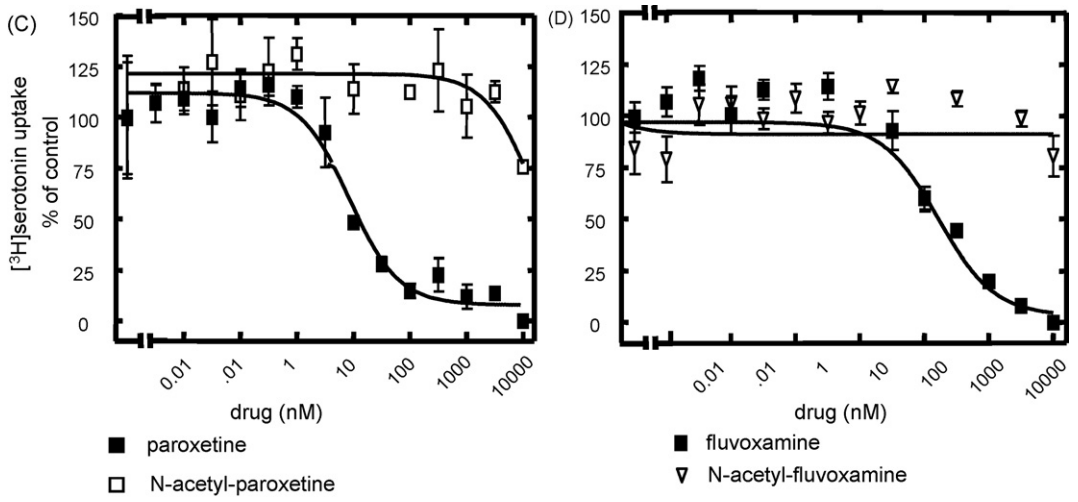

Fig. 6. (Continued).

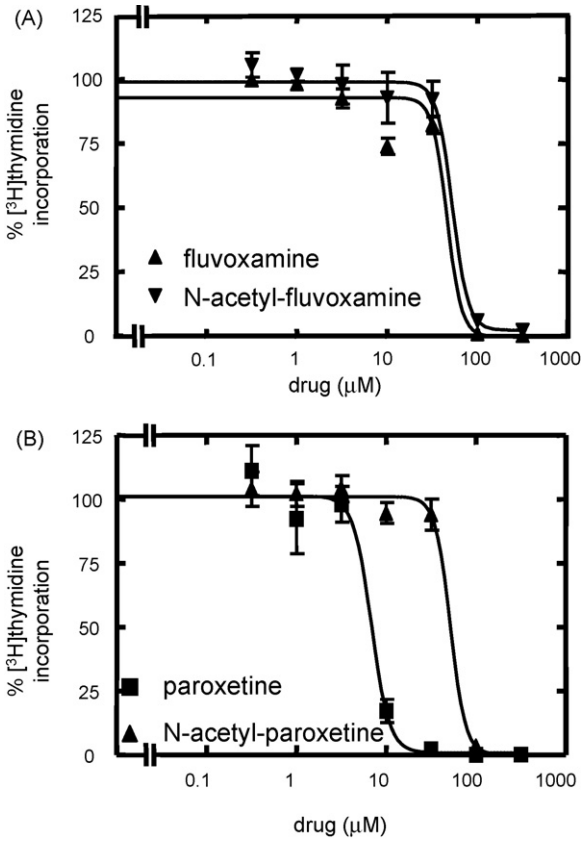

Fig. 7 – Inhibition by acetylated fluvoxamine (A) and paroxetine (B) of [^{3}H]thymidine incorporation by a murine Eμ-myc tumor cell line. Established murine Eμ-myc tumor cells (10^5 cells/well) were seeded in 96 well plates and incubated with the indicated concentrations of fluvoxamine and N-acetyl-fluvoxamine acetate (A) or paroxetine and N-acetyl-paroxetine (B). After 24 h cells were lysed, the radioactively labeled DNA was trapped on filter mats and [^{3}H]thymidine incorporation was determined by liquid scintillation counting. Data shown represent means ± S.E.M. (n = 3).

In contrast, the concentration–response curve for inhibition of [^{3}H]serotonin uptake was consistent with a simple stoichiometric interaction governed by the law of mass action. Based on these observations, we therefore conclude that at concentrations in the μM range, SSRIs interact with a (or several) ubiquitously expressed target(s); thereby these compounds inhibit the proliferation of and kill a large number of mammalian cells. This conclusion is also supported by the finding that – in this concentration range (10–300 μM) – many SSRIs (i.e., fluoxetine, paroxetine, fluvoxamine, citalopram and sertraline) are also effective spermicides [21]; this toxic action is apparently not confined to mammalian cells because some SSRIs also kill *Trichomonas vaginalis* [21].

We used two assays to detect the cytotoxic effects of SSRIs, namely inhibition of [^{3}H]thymidine incorporation and staining with annexin V. The latter assay monitors one of the earliest consequences triggered by programmed cell death, namely the inhibition of flippase and activation of scramblase activity, which results in the appearance of phosphatidylethanolamine and phosphatidylserine on the outer leaflet of the plasma membrane [22–24]. The [^{3}H]thymidine incorporation assay monitors the capacity of cells to enter the S-phase of the cell cycle and hence the proliferative rather than the apoptotic response of cells. Nevertheless, compounds that trigger apoptosis are also expected to cause a decline in [^{3}H]thymidine incorporation, because activation of the apoptotic program in a given cell precludes its recruitment into the cell cycle. Inhibition of [^{3}H]thymidine incorporation and the pro-apoptotic action of SSRIs occurred over a similar concentration range and showed similar steep concentration–response curves. Thus, inhibition of S-phase entry may have indirectly resulted from the pro-apoptotic action of SSRIs. Alternatively, it is conceivable that SSRIs have two independent effects, i.e., inhibition of S-phase entry and induction of apoptosis result from the activation of two independent signaling pathways. We did not design experiments to differentiate between these two possibilities. Given the lack of selectivity in the cytotoxic action of SSRIs, it appears of modest interest to unravel the molecular details.

In Burkitt lymphoma, the initiating event is thought to be the deregulated expression of c-*MYC*. Overexpression of c-*MYC* is caused by a chromosomal translocation the most common of which recombines the *MYC* gene (residing on chromosome 8; 8q24) with the immunoglobulin heavy chain (*IGH*) locus (on chromosome 14; 14q32) [25,26]. Typically, the entire *MYC* gene (including the first non-coding exon and the two major promoters) is inserted into the joining region J_H of the *IGH* locus region adjacent to the intronic heavy chain enhancer Eμ [27]. This situation is recapitulated in the Eμ-myc transgenic mouse [12], which we therefore used as a murine model system mimicking myc-driven transformation of human germinal B-cells. Tumor cells derived from Eμ-myc transgenic mice were most sensitive to the inhibitory action of SSRIs (IC_{50} for paroxetine ~5 μM). In contrast, the T cell leukaemia line Jurkat was less susceptible to the cytotoxic effect of paroxetine (IC_{50} ~ 110 μM). It may therefore be argued that our data confirm the original interpretation [6] that Burkitt lymphoma cells are exquisitely sensitive but Jurkat and other T cell lines are resistant to SSRIs. There are, however, additional points that need to be taken into account: (i) the previous studies had used quiescent lymphocytes for control purpose and found them to be resistant to SSRIs. We investigated proliferating murine primary splenocytes; the difference in potency of SSRIs between these cells and Eμ-myc-positive tumor cells was modest. The discrepancy between our observations and those of Gordon and co-workers [6,7] may be due to the fact that proliferating cells are more prone to undergo apoptosis than quiescent cells. From a therapeutic perspective, however, the cytotoxic action of SSRIs on proliferating lymphocytes is relevant, because expansion of lymphocyte populations is necessary to mount an effective immune response. (ii) The concentration–response curves for the SSRI-induced cytotoxic action were very steep (slope factors/Hill coefficients >3). Thus, the effect is apparently contingent on the accumulation of a signal which triggers the cytotoxic action. Because of this threshold phenomenon, we surmised that upon prolonged incubation, less susceptible cells would eventually also become more susceptible. This conjecture was verified for three representative cell lines: in each case, the IC_{50} for growth inhibition was lower at long incubation times. There was one notable exception: the concentration curve was shallow (i.e., slope factor/Hill coefficient ~1), if SSRIs were tested on primary tumor cells harvested from the bone marrow of Eμ-myc transgenic mice (Fig. 3A, open squares). We consider it unlikely that this discrepancy arises from the presence of a specific target site in these cells that allows for a stoichiometric interaction of SSRIs. The more parsimonious and hence plausible explanation is to consider the fact that this cell preparation consists of a heterogeneous cell population. Accordingly, some tumor cells are highly sensitive to inhibition by SSRIs while others are resistant. This interpretation is supported by the observation that the concentration–response curve for SSRIs was steep, if assessed with an established Eμ-myc tumor cell line (Fig. 1A, open squares). Thus, at the present stage, there is little evidence to justify the use of SSRIs in Burkitt lymphoma and other B-lymphoid malignancies. Firstly, concentrations required to elicit a direct toxic action are far above the therapeutic range. SSRIs can *per se* lead to a potentially life-threatening serotonin syndrome. Secondly, as amply demonstrated in the current work, the compounds lack selectivity and are expected to affect many rapidly proliferating cells. It is, however, conceivable that SSRIs may sensitize many different tumor cells to the actions of cytotoxic drugs and that these effects may be achieved at concentrations, which are achieved at therapeutic doses.

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REFERENCES

[1] van den Bosch CA. Is endemic Burkitt's lymphoma an alliance between three infections and a tumour promoter? Lancet Oncol 2004;5:738–46.
[2] Rochford R, Cannon MJ, Moormann AM. Endemic Burkitt's lymphoma: a polymicrobial disease? Nat Rev Microbiol 2005;3:182–7.
[3] Carbone A. Emerging pathways in the development of AIDS-related lymphomas. Lancet Oncol 2003;4:22–9.
[4] Meremikwu MM, Ehiri JE, Nkanga DG, Udoh EE, Ikpatt OF, Alaje EO. Socioeconomic constraints to effective management of Burkitt's lymphoma in south-eastern Nigeria. Trop Med Int Health 2005;10:92–8.
[5] Serafeim A, Grafton G, Chamba A, Gregory CD, Blakely RD, Bowery NG, et al. 5-Hydroxytryptamine drives apoptosis in biopsy-like Burkitt lymphoma cells: reversal by selective serotonin reuptake inhibitors. Blood 2002;99:2545–53.
[6] Serafeim A, Holder MJ, Grafton G, Chamba A, Drayson MT, Luong QT, et al. Selective serotonin reuptake inhibitors directly signal for apoptosis in biopsy-like Burkitt lymphoma cells. Blood 2003;101:3212–9.
[7] Meredith EJ, Holder MJ, Chamba A, Challa A, Drake-Lee A, Bunce. et al. The serotonin transporter (SLC6A4) is present in B-cell clones of diverse malignant origin: probing a potential anti-tumor target for psychotropics. FASEB J 2005;19:1187–9.
[8] Marti RM, Wasik MA, Kadin ME. Constitutive secretion of GM-CSF by three different cell lines derived from a single patient with a progressive cutaneous lymphoproliferative disorder. Cytokine 1996;8:323–9.
[9] Morikawa S, Tatsumi E, Baba M, Harada T, Yasuhira K. Two E-rosette-forming lymphoid cell lines. Int J Cancer 1978;21:166–70.
[10] Epstein AL, Kaplan HS. Biology of the human malignant lymphomas. I: Establishment in continuous cell culture and heterotransplantation of diffuse histiocytic lymphomas. Cancer 1974;34:1851–72.
[11] Stoiber D, Kovacic B, Schuster C, Schellack C, Karaghiosoff M, Kreibich R, et al. TYK2 is a key regulator of the surveillance of B lymphoid tumors. J Clin Invest 2004;114:1650–8.
[12] Adams JM, Harris AW, Pinkert CA, Corcoran LM, Alexander WS, Cory S, et al. The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature 1985;318:533–8.
[13] Hilber B, Scholze P, Dorostkar MM, Sandtner W, Holy M, Boehm S, et al. Serotonin-transporter mediated efflux: a

pharmacological analysis of amphetamines and non-amphetamines. Neuropharmacology 2005;49:811–9.
[14] Scholze P, Norregaard L, Singer EA, Freissmuth M, Gether U, Sitte HH. The role of zinc ions in reverse transport mediated by monoamine transporters. J Biol Chem 2002;277:21505–13.
[15] Chanrion B, Mannoury la Cour C, Bertaso F, Lerner-Natoli M, Freissmuth M, Millan MJ, et al. Physical interaction between the serotonin transporter and neuronal nitric oxide synthase underlies reciprocal modulation of their activity. Proc Natl Acad Sci USA 2007;104:8119–224.
[16] Gottesman MM. Chinese hamster ovary cells. Methods Enzymol 1987;151:3–8.
[17] Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 1977;36:59–74.
[18] Gordon J, Barnes NM. Lymphocytes transport serotonin and dopamine: agony or ecstasy? Trends Immunol 2003;24:438–43.
[19] Pratuangdejkul J, Schneider B, Jaudon P, Rosilio V, Baudoin E, Loric S, et al. Definition of an uptake pharmacophore of the serotonin transporter through 3D-QSAR analysis. Curr Med Chem 2005;12:2393–410.
[20] Keverline-Frantz KI, Boja JW, Kuhar MJ, Abraham P, Burgess JP, Lewin AH, et al. Synthesis and ligand binding of tropane ring analogues of paroxetine. J Med Chem 1998;41:247–57.
[21] Kiran Kumar ST, Sharma VL, Kumar M, Shukla PK, Tiwari P, Jain RK, et al. Synthesis of benzenepropanamine analogues as non-detergent spermicides with antitrichomonas and anticandida activities. Bioorg Med Chem 2006;14:6593–600.
[22] Verhoven B, Schlegel RA, Williamson P. Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. J Exp Med 1995;182:1597–601.
[23] Verhoven B, Krahling S, Schlegel RA, Williamson P. Regulation of phosphatidylserine exposure and phagocytosis of apoptotic T lymphocytes. Cell Death Differ 1999;6:262–70.
[24] Bratton DL, Fadok VA, Richter DA, Kailey JM, Guthrie LA, Henson PM. Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. J Biol Chem 1997;272:26159–65.
[25] Dalla-Favera R, Bregni M, Erikson J, Patterson D, Gallo RC, Croce CM. Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci USA 1982;79:7824–7.
[26] Taub R, Kirsch I, Morton C, Lenoir G, Swan D, Tronick S, et al. Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci USA 1982;79:7837–41.
[27] Boxer LM, Dang CV. Translocation involving c-myc and c-myc function. Oncogene 2001;20:5595–610.